Reference: *Biol. Bull.*, 138: 14–25. (February, 1970)

AN AUTORADIOGRAPHIC STUDY OF THE TRANSLOCATION AND UTILIZATION OF AMINO ACIDS BY STARFISH[1]

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In a series of previous reports (Ferguson, 1964ab, 1966, 1968a) experimental evidence has been presented which indicates that the circulation of fluid in the coelomic spaces, together with active cellular mechanisms of uptake and exchange, are largely responsible for the translocation of nutritive substances throughout the bodies of starfish. While such studies have provided considerable insight into these physiological processes, a great deal is still unknown about the functioning of the distributive systems in these animals. In particular, little direct evidence has been obtained on the movements of nutrients through the thick layers of connective tissue comprising the body wall, or of the conductive functions of the complex of structures located in the ambulacral region—the water vascular system, the so-called "hemal system," and the perihemal canals. Furthermore, the primary source of nutrition of the epidermis is still in doubt, as this tissue has been shown to be capable of taking considerable subsistence directly from the exterior (Ferguson, 1967ab, 1968b).

While these particular anatomical regions do not lend themselves well to most kinds of experimental study, it was felt that some understanding of the nutritive physiology of these parts could be obtained by using the technique of autoradiography to examine the movements into and the retention by these regions of radioactivity from C^{14}-labeled amino acids previously delivered by injection into the interior of the animals. The following will report the results of such a study.

Materials and Methods

Medium-size specimens of the starfish, *Echinaster echinophorus*, were collected from tidal grass flats near the southern shore of Tampa Bay. After being maintained in the laboratory for a few days, each was injected in the middle of the central disc with approximately 0.1 ml of filtered sea water containing 2–3 microcuries of C^{14}-labeled amino acid mixture (obtained from New England Nuclear Corporation of Boston, Massachusetts, and said to represent a synthetic *Chlorella* protein hydrolysate). In injecting the disc region, the bulk of the solution was delivered either into the lumen of the stomach or to the surrounding perivisceral coelom. While it was impossible to tell which at the time, the difference could easily be determined by the location of the radioactivity in the digestive glands demonstrated by the autoradiographs. Otherwise, the location of the injection seemed to make little difference in the results.

Immediately following the injection, the animals were rinsed several times in fresh sea water and then placed in tanks of sea water which was changed fre-

[1] Supported by NSF grant GB 6906.

quently, particularly during the first few hours. It was hoped that this procedure would prevent any of the labeled amino acid that might leak out of the injection injury from being taken up by the epidermis. Subsequent results verified the general success of this procedure, although in a few specimens some external uptake was detected. The animals were fed pieces of clam *ad libitum* while being retained.

At intervals after the injection of 1 hour, 24 hours, 20 days, 35 days, 55 days and 75 days, pairs of animals were sacrificed and two of their arms removed. These were cut in half and placed in one-half strength Bouin's solution for several days (this not only fixed the tissues, but also served to decalcify them). The tissues were then dehydrated in alcohols and embedded in paraffin under vacuum. Sections were cut at 10 microns thickness and prepared as Kodak AR-10 stripping film autoradiographs. Groups of slides were developed and studied (unstained) after exposure periods ranging from 1 month to a year. The autoradiographs were compared to other sections stained in Mallory's phosphotungstic acid (PTA) hematoxylin.

Observations

Much of the labeled amino acid administered in the disc was apparently rapidly translocated to various regions along the length of the arms and incorporated into insoluble material that could be visualized by the autoradiographic technique. Doubtless any remaining soluble compounds were lost during the processing of the sections or oxidized to CO_2 by the animals. Even in specimens sacrificed after just one hour, high retention of the tracer was observed in both the digestive glands and visceral peritoneum (Fig. 1). Particularly noteworthy was the uptake within the lining of the papulae extending through the body wall, and the area around the mesenteric supports. There was little difference in uptake between the proximal and the distal regions of the arms. Presumably the labeled amino acid was rapidly moved to all these places by ciliary currents maintained in the digestive glands and coelomic spaces. The existence of such currents have been documented in several species by Gemmill, 1915; Irving, 1924; Budington, 1942; Anderson, 1960; and others.

Amoebocytes located in all areas of the perivisceral coelom exhibited distinctive amounts of the label, although they generally did not take up and retain more of the C^{14}-amino acid than did the other cells exposed to the circulating fluids.

Much the same general distribution of labeled material as was found in the 1-hour specimens was also seen in those sacrificed at other time intervals (Figs. 2–6), along with a number of progressive changes. By 24 hours large quantities of retained labeled material had become located in the hemal system and associated connective tissue regions of the tube feet (Fig. 2) and by 5 days quantities of radioactive substances were seen dispersing in the connective tissue adjacent to the peritoneum and accumulating in a layer beneath the epidermis and in the outer regions of the large glands located in the dermis (Figs. 3, 7, and 8). Uptake by the lining cells of the tube feet was discernable at this time, probably indicating some translocation, *via* the water vascular fluid, from the very radioactive ampullar region. Labeled amoebocytes also began to appear in the body wall, water vascular system and various other places besides the perivisceral coelom. From 20 days on these dispersions continued with relative increases in radioactivity in most

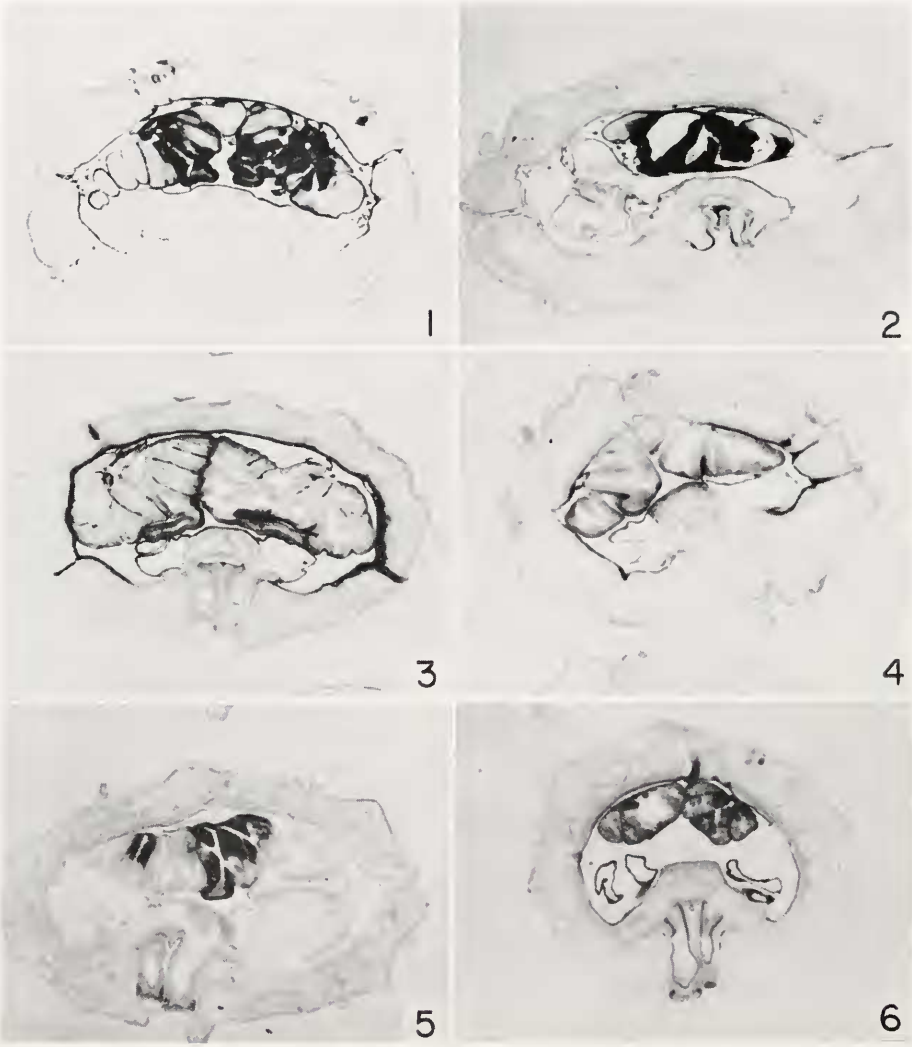


FIGURE 1. Unstained autoradiograph of arm section 1 hour after injection of C^{14}-amino acids. Note retention of radioactivity in tissues associated with perivisceral coelom. Slight darkening of epidermis is due to natural pigmentation and small amounts of tracer absorbed by this tissue directly from the exterior medium; 11 ×.

FIGURE 2. Section similar to that in Figure 1 from animal sacrificed 24 hours after injection. Note activity in hemal septum and connective tissue layer of tube feet; 10 ×.

FIGURE 3. Section similar to the previous ones from animal sacrificed 5 days after injection. Hemal septum is still radioactive. Some labeled amoebocytes are in the ampullae and the tube feet. Greater dispersion of radioactivity in the body wall is now discernable; 10 ×.

FIGURE 4. Section similar to the previous ones 20 days after injection. More dispersion of radioactivity through the dermis has occurred; 12 ×.

FIGURES 5 and 6. Sections similar to the previous ones from animals sacrificed at 55 and 75 days after injection. Much dispersion of radioactivity has occurred in the various tissues, but the most active areas are still the same as they were at 24 hours; 10 × and 14 ×.

areas of the body (Figs. 4, 5, and 6). These later changes were not dramatic, however, and most of the radioactivity remained in the same locations in which it was found at five days.

The 35-day, 55-day, and 75-day specimens seemed to require longer periods of exposure before satisfactory autoradiographs could be obtained, perhaps indicating metabolic utilization of the administered amino acid over the extended post-injection period.

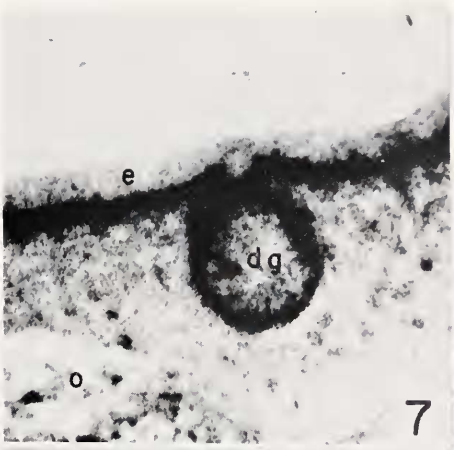
Intradermal translocation

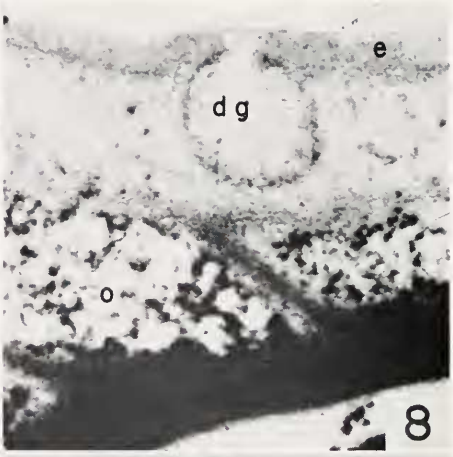
While at 24 hours practically no labeled material was detected in the body wall, outside the peritoneum, significant translocation through the dermis was quite apparent in specimens sacrificed after 5 days (Figs. 7 and 8). In these animals radioactive material has moved from the peritoneum, through the fibrous meshwork of the connective tissue, all the way to the area beneath the epidermis. In Figure 8 the large quantities of radioactivity dispersing outward from the peritoneum are especially evident. The retention of the labeled material in the connective tissue area indicates that the administered C^{14}-amino acids have been synthesized into insoluble elements of the connective tissue, particularly mucopolysaccharide ground substance and collagenous fibers (see Ferguson, 1960). The retention of the labeled substance is greatest in the areas adjacent to the peritoneum and the epidermis. Study of stained sections indicate that both these areas contain numerous cells of various types and are probably prime locations for the production of connective tissue substance.

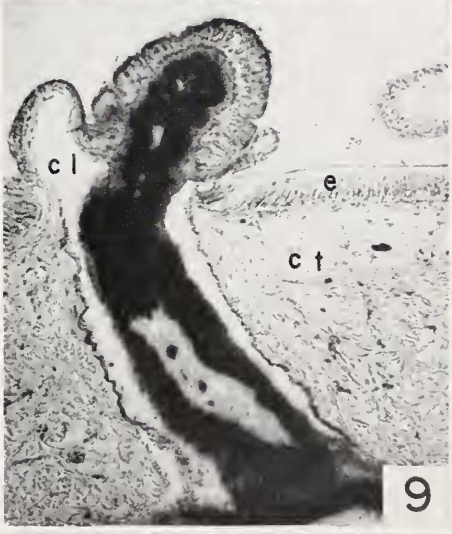
Uptake of tracer by individual cells could be detected freely throughout the dermal area in the 5-day specimens. There was an especially significant uptake by the osteocytes in all areas of the ossicles. As the solid nature of the ossicles would probably restrict the diffusion of the labeled nutrients into them, it seems likely that nutrients reach the interior of these structures by cell to cell transport. There was also a considerable uptake in the secretory cells of the large dermal glands, which was still evident even at 75 days (Fig. 10). This observation confirms that these organs obtain most of their raw materials from nutrient sources within the body.

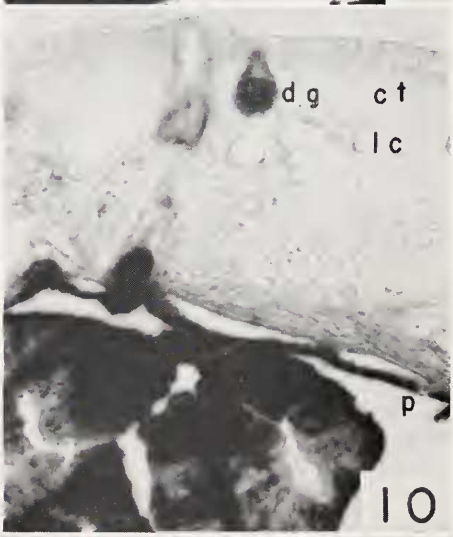
While considerable radioactivity could be seen in the dermal region of most specimens after 5 days, practically no label was detected in their epidermis (Fig. 7 and others). As the great affinity of the epidermis for free exogenous nutrients has already been well documented (Ferguson, 1967b, 1968b), this observation strongly supports the hypothesis that there is some sort of functional diffusion barrier against amino acids, their products, and probably other nutrients located beneath the epidermis. Indeed, the distribution of radioactivity shown in Figure 7 remarkably complements that of previously published autoradiographs (Ferguson, 1967b) of animals exposed to C^{14}-labeled amino acids in their external media. Such a diffusion barrier would largely prevent losses of soluble nutrients from the animals, and require that the epidermis obtain the bulk of its nutrition from exogenous sources.

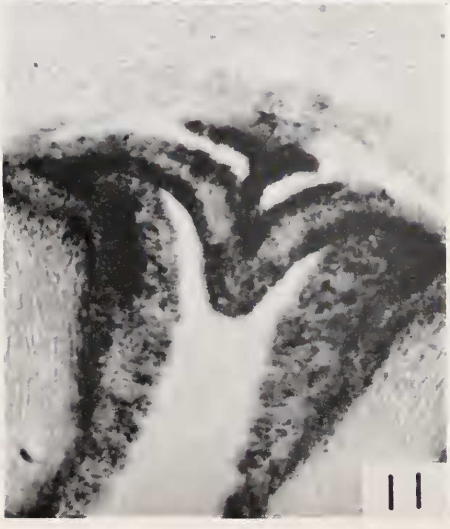
Running more or less randomly as a network throughout the outer regions of the body wall of most specimens are open lacunar channels (dermal spaces). One might expect these, together with the concentric lacunar spaces associated with

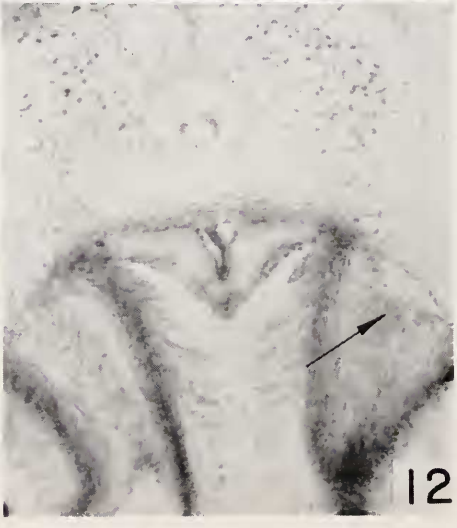












FIGURES 7–12.

the papulae (Fig. 9), to serve as channels for the dispersal of nutrients. In general, however, very little increased radioactivity could be detected in the vicinity of these areas in any of the sections. Thus, if they do have a nutrient transport function, it was not made evident by the limitations of the technique employed. Their general organization, however, would suggest a significant role in respiration.

Hemal system function

The appearance of large quantities of radioactivity in the hemal system and some of the adjacent lateral regions, beginning with the 24-hour specimens, was a startling observation and demanded careful study. A transport function was ascribed to this system by a number of workers of the last century and especially championed by Cuènot (1887). More recently, however, it has been widely believed that the system, in asteroids at least, is largely vestigial and that any transport of nutrients required by the ambulacral area would be accomplished in the adjacent perihemal spaces and water vascular system. An alternative function for the hemal system was reported by Unger (1962), who indicated that it transported neurosecretory products from the radial nerve cord to the water vascular system. He diagramed the hemal tissue as hollow vessels opening into the transverse water vascular canals.

As stated, the region of the hemal septum and its connecting structures became intensely radioactive beginning with the 24-hour specimens (Fig. 2), and they retained considerable activity even after 35 days (Fig. 11). After 55 and 75 days (Fig. 12), the intensity of the radioactivity was somewhat diminished, as it was throughout most regions of these animals.

Interestingly, the activity found in the hemal system and the lateral regions was not uniform along the length of the arms. Rather, it would build up over a number of serial sections and then decrease again to negligible amounts before beginning to build back up again. For this reason it is unlikely that the observed activity represented organic material transported centrifugally from the disc, as might be expected if the system were functioning as a simple vascular system.

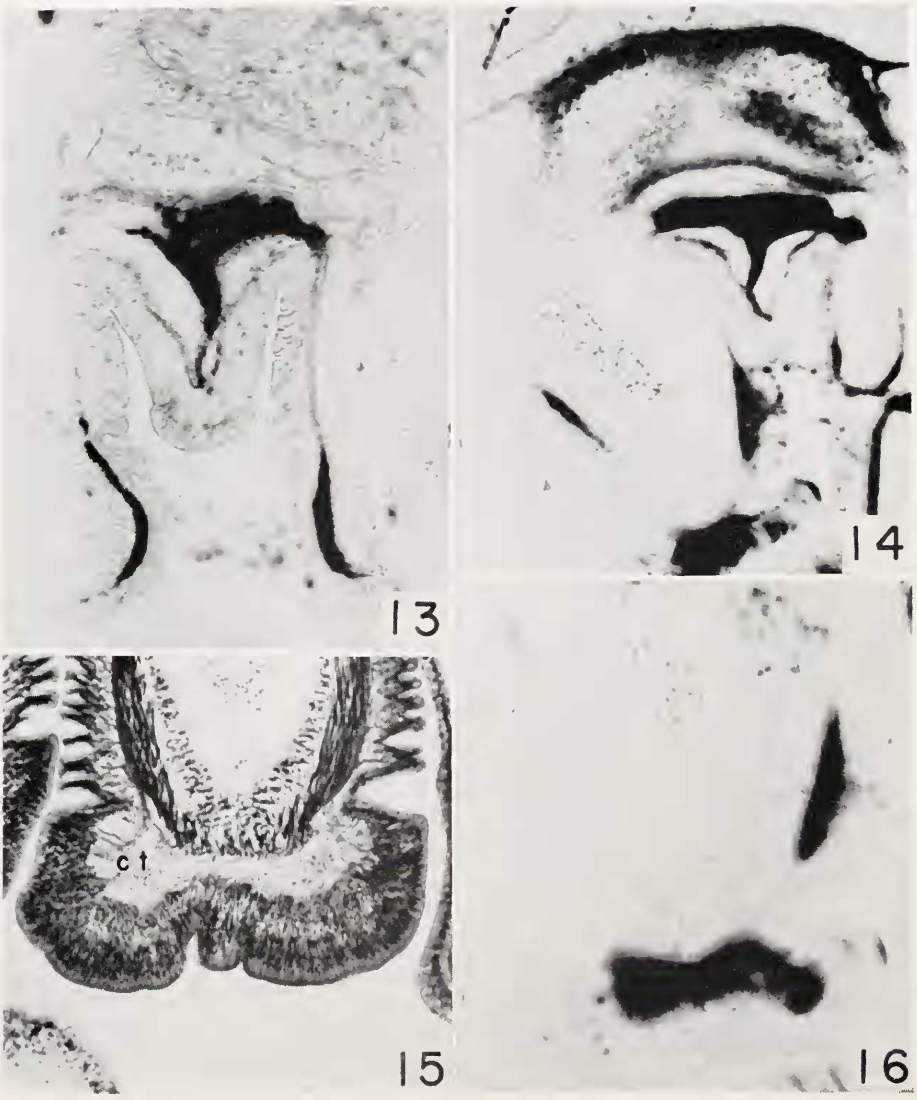
The radioactivity found in the hemal septum could be traced as being contiguous, *via* the transverse hemal processes, with large quantities of labeled substance found in sheets of connective tissue located in the middle layer of the tube

FIGURES 7 and 8. Autoradiographs of arm sections of animal 5 days after injection. Note how epidermis (e) remains almost free of the radioactivity found in the subepidermal region, and large dermal gland (dg). In addition to labeled material dispersing through the body wall, many radioactive cells may be seen including osteocytes (o) in the ossicles; 110 × and 85 ×.

FIGURE 9. Autoradiograph of section through papula of specimen 24 hours after injection. While peritoneal lining of this structure is very radioactive, no activity has yet reached the body wall connective tissue (ct) or epidermis (e); (cl = circumferential lacuna); 100 ×.

FIGURE 10. Autoradiograph of section of aboral body wall from animal 75 days after injection. While radioactivity may be seen dispersed in the connective tissue (ct) and large dermal gland (dg), there is no significant increase in its level in the vicinity of the lacunar channels (lc); (p = peritoneum); 70 ×.

FIGURES 11 and 12. Autoradiographs of sections through ambulacral areas of animals sacrificed 35 and 75 days after injection. Some progressive dispersions of radioactivity from the hemal structures and tube foot connective tissue may be detected. In Figure 12 the tube foot lining cells (arrow) have apparently taken up radioactive material from the water canals; 110 × and 70 ×.



FIGURES 13 and 14. Autoradiographs of nearly adjacent sections through ambulacral area of animal sacrificed 24 hours after injection. Note the rather specific retention of the label in the hemal tissue and associated connective tissue layer of tube feet. Unlabeled amoebocytes are located in the bottom of left tube foot and radial water canal; 100 × and 70 ×.

FIGURE 15. Section of tube foot stained with PTA hematoxylin. Note how the connective tissue layer (ct) fans out into the sucker; 110 ×.

FIGURE 16. Autoradiograph of section similar to that in Figure 15 showing radioactive material in connective tissue layers 24 hours after injection; 100 ×.

foot walls (Figs. 13 and 14). This connective tissue is located between the longitudinal muscle cells and the elongate epidermal cells of these structures. Above the sucker, the connective tissue may be seen to "fan out" in stained sections (Fig. 15), and this area also collects radioactive material (Fig. 16). Some further dispersion of the activity amongst the epidermal cells of the sucker was observed in specimens kept for the longer periods of time (Figs. 5 and 6).

While in the 24-hour specimens some uptake of the tracer was seen occasionally in Lange's nerve and other areas exposed to the perihemal canals (Fig. 14), by far the major portion of the activity was found in the hemal structures and the connective tissue layers of the tube feet just described. In specimens retained for longer periods, slightly greater dispersion of activity into adjacent areas could be detected (Figs. 11 and 12).

The histological structure of the hemal system of these animals is very difficult to interpret. Study of stained sections (Figs. 17 and 18) indicate that it consists of a dense network of connective tissue and channels filled with numerous fixed cells, amoebocytes, and thick ground substance. In general appearance its structure is, on a smaller scale, not too dissimilar from that of the echinoid axial organ described by Millott (1966). As the ground substance stains intensely with the periodic acid-Schiff reaction (unaffected by salivary digestion), and with protein stains such as bromphenol blue, it is probably a mucopolysaccharide.

In the region in which the transverse water vascular canal passes out to connect with the tube foot (Fig. 18), the hemal septum also fans out and connects with the connective tissue of the wall of the transverse water vascular canal and sheets of connective tissue in the wall of the tube foot. In my sections there is no indication of a continuous hemal canal that might connect with this water vessel as described by Unger (1962). The spaces seen in the hemal tissue can usually be traced to openings into the perihemal canals.

A distinctive feature of the hemal system is the large number of amoebocytes usually found in and around it. They often fill up the hemal channels and particularly may be found in the perihemal space above the hemal septum where it fans out to make its lateral connections (Fig. 17). Possibly these cells could be responsible for bringing the labeled substance to the hemal tissue. In the 24-hour specimens labeled amoebocytes are found in the perihemal canals in the vicinity of the highly radioactive areas of the hemal septum. Few such cells, however, may be located elsewhere in the ambulacral area. In particular, numerous unlabeled amoebocytes are found in all areas of the water vascular system (Figs. 13, 14 and 16). While it is possible that amoebocytes could transport labeled amino acids in a soluble form which would not be preserved by the technique employed, other evidence, particularly the presence of heavily labeled amoebocytes elsewhere, would indicate that transport of such material by these cells is probably not significant.

If the amoebocytes do not bring the bulk of the radioactivity to the hemal septum, where does this tissue obtain it? The probably answer may be found by tracing the lateral extensions of the perihemal canals in their circuit around the tube feet (Fig. 19). It is discovered that they make a rather lengthy juxtaposition with the perivisceral coelom (Fig. 20). Detailed study of this region also indicates that the peritoneal lining changes from its general columnar character

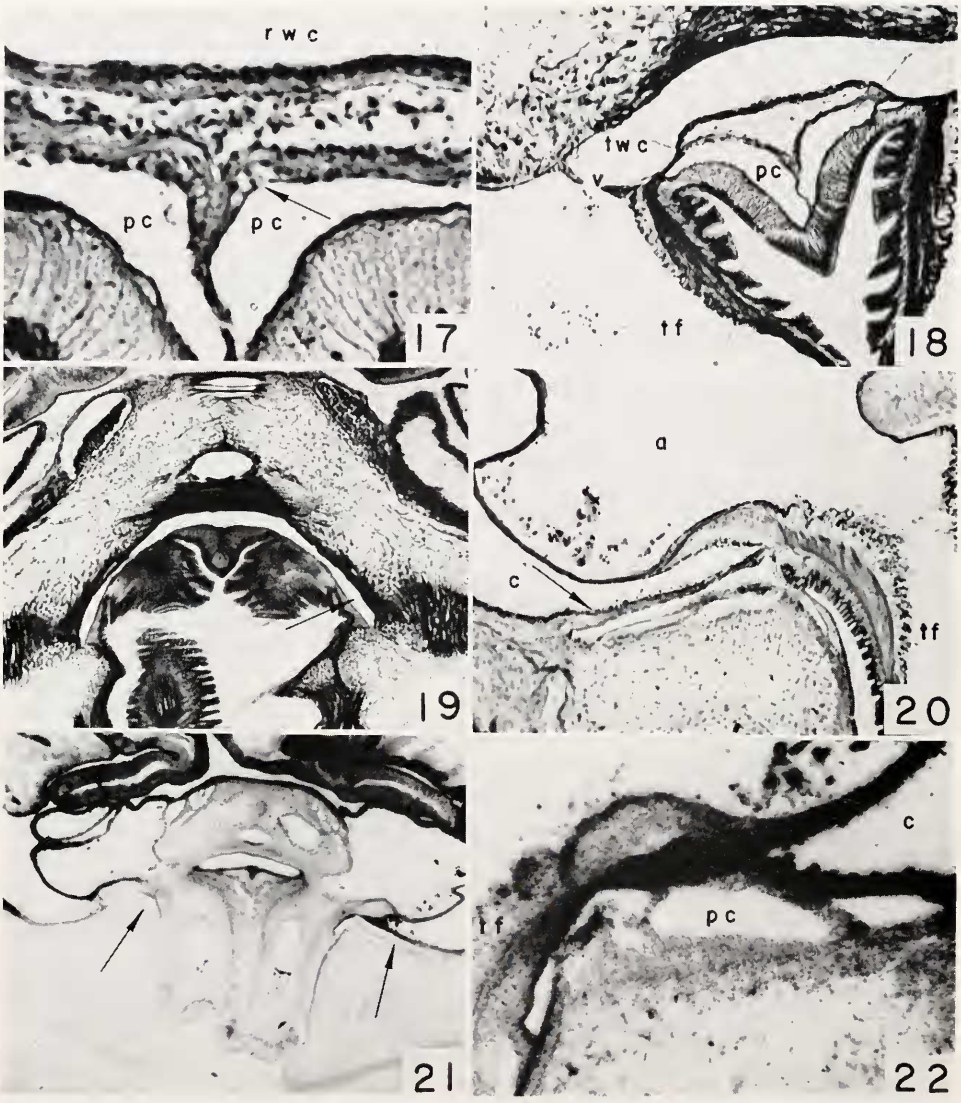


FIGURE 17. Section through hemal septum making its lateral connections; PTA hematoxylin. Note hemal channels (arrows) which open into perihemal canals (pc) and numerous amoebocytes, especially above hemal system; (rwc = radial water canal); 310 ×.

FIGURE 18. Section through ambulacral area showing lateral connections of the hemal septum with the wall of the transverse water canal (twc) and tube foot connective tissue layer; PTA hematoxylin (v = valve; tf = tube foot; pc = perihemal canal); 100 ×.

FIGURE 19. PTA hematoxylin stained section showing lateral extension of the perihemal canals (arrow); 40 ×.

FIGURE 20. PTA hematoxylin stained section showing part of the juxtaposition of the perihemal canal and the perivisceral coelom (c). The peritoneum becomes thinner in this region (arrow); (a = ampulla; tf = tube foot); 310 ×.

to a more cuboidal or squamous form. In some sections (Fig. 21 and 22) the whole region appears very radioactive.

It is, then, highly possible, that soluble nutritive substances are transported between the two cavities in these regions. They are circulated through the perihemal canals, and efficiently taken up by the hemal septum, particularly as the fluid percolates through its many canals. Here the soluble materials are reduced to an insoluble form (probably a mucopolysaccharide), which is transported in loose connective tissue sheets to the distal ends of the tube feet where it appears to be dispersed in the sucker region.

Discussion

While the present study represents an experimental situation, there is much reason to believe that it accurately reflects the normal functioning of the animals. Small quantities of a number of different amino acids may always be detected in the coelomic fluid of *Echinaster* (author's observation). The addition, then, of small amounts of these substances (in a labeled form) should make little biological difference. Indeed, previous work (Ferguson, 1964ab, 1968a) has demonstrated how such dissolved nutrients, in spite of their low concentrations, probably play a major role in the uniform distribution of nutritive materials throughout the body.

As a result of this study, it is now possible to formulate a much more complete concept of how cells in various isolated regions of the animals obtain their subsistence. The basic theory is as follows (subject to further modification as additional data is obtained): Nutritional substances are actively taken up from the environment by ingestion through the mouth into the digestive glands, and by direct absorption (particularly of free amino acids and sugars) into exposed epidermal cells. The material obtained by the latter is used exclusively by them and is not passed on to the more internal parts in appreciable quantities, probably because of the presence of a diffusion barrier (See Ferguson, 1967ab, 1968b). These epidermal cells may, however, release some material back into the environment.

The material taken into the digestive glands is digested, stored, and slowly released to the circulating coelomic fluid, which transports it to the major "core" regions of the body. All the cells (including those of the digestive glands) lining the coelomic cavity take up and release these products in an apparent exchange process.

Nutrients taken up by the somatic peritoneum are allowed to diffuse through the connective tissue network or possibly are converted to a viscous, insoluble ground substance which can do that. In either case, the translocation across the dermal layer may be quite rapid, and active metabolic areas beneath the epidermis (including the large dermal gland cells) do not suffer from nutritional lack. The

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Figure 21. Autoradiograph of section from animal 5 days after injection. Note association of perihemal canals with perivisceral coelom (arrows); 25 ×.

Figure 22. More magnified view of right portion of Figure 21 to show association of perihemal canal (pc) and perivisceral coelom (c); (tf = tube foot); 310 ×.

nutritive material is largely blocked from entering the epidermis and leaving the body by the diffusion barrier located beneath this tissue.

Osteocytes, which form an interconnecting network throughout the ossicles, also are able to efficiently distribute nutrients amongst themselves, apparently by cell to cell transfer.

Cells located in the ampullae pass some of the nutritional products which they take up from the perivisceral coelom into the circulating fluid of the water vascular system. From here they may be taken up and utilized, particularly by the lining cells and muscle cells of the tube feet. Cells in certain specialized areas of the somatic peritoneum, lateral to the ampullae, similarly pass nutritive materials into the perihemal canals where they may be utilized by the cells lying adjacent to this compartment (particularly certain muscle and nervous elements). Much of the material appearing in the perihemal canals, however, is filtered out by the hemal structures and synthesized into insoluble compounds (apparently mucopolysaccharides) which are distributed through the connective tissue layer of the tube feet to the sucker region. Here they might be used as adhesive substances or for other purposes.

In their sojourns throughout the body, amoebocytes also pick up considerable quantities of nutritive materials. While these materials are generally retained in an insoluble form within the cytoplasm, the amoebocytes may, perhaps, disperse them in outlying regions, supplementing the other translocative systems.

Obviously, there is much more to be learned about the nutritional physiology of these animals. Little is yet known, for example, about the function of the axial organ and other hemal structures of the disc region. Application of techniques similar to those used in this study, however, should provide much useful insight into the function of these structures. Furthermore, the specificity with which the label was taken up by certain areas of the hemal system and tube feet in the present work suggests the possibility of biochemically identifying the substances being produced and transported by these regions using extraction and analytical procedures.

Summary

1. Specimens of the starfish, *Echinaster echinophorus*, were injected in the disc region with small quantities of C^{14}-labeled amino acid mixture. After periods ranging from 1 hour to 75 days they were sacrificed and histological sections of their arms prepared as stripping film autoradiographs.

2. By 1 hour after the injection bound radioactive material was detected in most tissues in the proximity of the coelomic cavity, and while translocations from these areas were noted, most of the labeled material remained in these areas even after 75 days.

3. By 24 hours large quantities of radioactivity were found spasmodically in the hemal septum and traced dispersing outward into the connective tissue layer of the tube feet and the suckers. It is believed that this material was transported from the peri-visceral coelom *via* the perihemal canals and fixed as an insoluble mucopolysaccharide by the cells of the hemal septum.

4. By 5 days significant dispersion of radioactive material through all regions of the body wall was evident. Strong retention of the radioactive material was

found in the subepidermal layer, the large demal glands, osteocytes, and in the connective tissue layers adjacent to the somatic peritoneum.

5. As no sign of movement of radioactivity into the epidermis was seen in any of the sections, it is concluded that a previously theorized diffusion barrier beneath this layer must exist, and that epidermal cells obtain most of their nutrition from external sources.

6. Judging by their distribution in the various sections, it seems possible that the amoebocytes play a minor, purely secondary role in the dispersion of nutritive substances throughout the body.

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